THE EFFECT OF LEUCOGENENOL AND CHEMOTHERAPY ON B AND T LYMPHOCYTE POPULATIONS IN MICE INFECTED WITH FRIEND VIRUS LEUKEMIA

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The problem. These experiments were designed to determine the effects of the immunostimulant leucogenenol and the combination chemotherapy of arabinosyl cytosine and vinblastine on the B and T lymphocyte populations of mice infected with Friend virus leukemia.

Procedure. Isolated lymphocyte populations were quantitated using a cytotoxic assay for T lymphs and a direct immunofluorescent technique for B lymphs. Spleen and liver weight to total body weight ratios, survival times, peripheral nucleated cell counts and peripheral white blood cell differentials were also monitored to show the progression of the FV leukemia and the effects of leucogenenol and chemotherapy.

Findings. The effect of the leucogenenol was to initially elevate the B and T lymphoid cell numbers of the peripheral circulation significantly over the untreated controls. Increases were also noted between FV infected mice and those FV-infected mice that were given leucogenenol. The effects of the drugs were to lower the peripheral lymphoid cell numbers and significantly reduce the spleen and liver weight to total body weight ratios. Mixed effects were noted in all groups and at the various time intervals in the percentage of B and T lymphs in the spleen and lymph node cell suspensions.

Conclusion. The effect of leucogenenol seems to be the stimulation of both B and T lymphocyte stem cell lines. This is most clearly shown by the increase in the number of identifiable B and T lymphocytes in the peripheral blood. The effect of the drugs was to offset the effects of the FV. The addition of leucogenenol to the therapy seems to work in opposition to the drugs by reducing their leucopenic affects.

Recommendations. More studies on the cellular action of leucogenenol are necessary. The combination of leucogenenol and an anti-B-lymphocyte serum may prove effective in counteracting virus-induced leukemias. Further studies using leucogenenol on solid tumors such as sarcomas and carcinomas should be done as in these cases, the neoplasias do not directly affect the hemapoietic process.

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INTRODUCTION AND REVIEW OF THE LITERATURE

Surgery, radiation and chemotherapy are today the most widely used means of clinically treating cancers. All present therapeutic regimens are immunosuppressive in some degree to the host. Although combination drug chemotherapy is today widely used in cancer treatment, it has many deleterious effects. First, there is the toxicity of the drugs to the host. Many of the drugs themselves are oncogenic or they may effectively potentiate natural carcinogens already present in the host. The drugs may cripple the host's specific immune surveillance system (i.e., T or thymus lymphocyte subpopulation) and finally it can lead to a general weakening of the host's nonspecific defense mechanisms (Penn and Starzi, 1973). Researchers are now turning to various immunotherapeutic approaches in the hope of lessening the deleterious effects of the other cancer therapies by either lowering dosages or shortening treatment schedules. It is postulated that a stimulated immune system may effectively eliminate all tumor cells from the host, something other methods of therapy rarely do. It was the purpose of this study to examine a possible nonspecific immunotherapeutic

agent, leucogenenol, and to evaluate its effects on the host's immune system by following the changes in the Bursa or B and Thymus or T lymphocyte populations.

Friend Virus

In 1957, Charlotte Friend found in the course of examining a cell-free extract of an Ehrlich ascites tumor, an agent capable of producing leukemia in mice. She described the following physical characteristics of the agent (Friend, 1957). It was found to be still active after six months storage at -70°C or after eleven days at 4°C. It could be lyophilized and stored in this form for at least three months. Its infectivity was destroyed by heating at 56°C for 30 minutes or overnight exposure to ether or a 1:200 dilution of formalin.

Friend virus (FV), named after its discoverer, Dr. Charlotte Friend, is a medium sized RNA spherical virus that appears in two distinct forms, types A and C (Bernhard and Guerin, 1958). The A particle is generally found in the cytoplasm of infected cells, although it is occasionally found extracellularly (deHarven, E. and C. Friend, 1965). Electron microscope studies of the A particle show it to be doughnut-shaped with less electron density in the middle

than on the periphery. Its diameter averages 87 mμ and it may or may not be enveloped (deHarven, E. and C. Friend, 1958). Studies of the C type particle show it to have maximum electron density in the center. The dense area is called the nucleoid and it contains the genome of the virus. The particle has a diameter of 100 mμ and is encased in an envelope (deHarven, E. and C. Friend, 1960a). The C type particles are localized in cytoplasmic vacuoles, can be seen budding from plasma membranes and more frequently in extracellular spaces. They are never truly intracytoplasmic and for this reason are often considered to be a mature form of the A particles (deHarven, E. and C. Friend, 1960b). The C particle envelope is acquired through a budding and transformation sequence. The budding takes place both at the plasma membranes and in cytoplasmic vacuoles. The particles are seen in intimate contact with the cellular membranes of infected cells and budding from these membranes forms the C particle envelope. Acquisition of this cellular membrane is responsible for the ether sensitivity of the virus (deHarven, E. and C. Friend, 1960a). FV may replicate in a cell without transforming it (eg. megakaryocytes), or transform a cell without necessarily replicating in it (eg. reticulum cell), (deHarven, E. and C. Friend, 1960b).

FV has been described as a leukemia of the erythroid-myeloid series (Metcalf et al., 1959). There are several strains of FV that may alter the course of the disease, especially the blood picture (Mirand, 1965). The hematologic manifestations and symptoms mentioned will be for the anemia-producing strain of FV used in these investigations. The disease is characterized by neoplastic proliferation of reticulum cells with associated erythroblastosis and lymphocytosis. A progressive leukocytosis occurs by the second day after infection and reaches levels of 15,000-30,000/cu mm in 7-14 days. Cell counts in excess of 100,000/cu mm can be found in the terminal phase of the disease. The leukocytosis is due predominantly to an increase in lymphocytes. Polymorphonuclear neutrophil (PMN) levels remain near normal throughout the disease (Metcalf et al., 1959). The peripheral blood is characterized by the presence of a large nucleated cell called the "Friend Cell" (Friend, 1957).

A detailed study of the pathogenesis of FV disease by Metcalf et al. (1959) revealed that the reticulum cells of the spleen seem to be the primary sites of FV infection. As early as three days after infection, the reticulum cells of the red pulp of the spleen show signs of neoplastic

proliferation. These neoplastic cells enter the venous sinuses and are carried to the liver. At the same time, an accumulation of cells in the spleen causes rapid enlargement of the organ and in most cases, rupture in 10 to 30 days, resulting in death. When splenic rupture does not occur, hepatomegaly develops and death due to liver failure occurs in 50 to 80 days (Metcalf et al., 1959). Splenic enlargement is due to leukocyte and erythrocyte accumulation, neoplastic transformation of reticulum cells and the development of hemorrhagic necrosis (Elliott et al., 1970). The failure of the erythrocyte precursor cells to mature and the crowding out of normal erythroblastic cells in the bone marrow results in the anemia characteristic of the disease (Siegler and Rich, 1965).

The cells that FV preferentially replicates in has been a source of controversy. Early work by Siegler and Rich (1965) and Boiron et al. (1965) suggested the site of FV proliferation to be proerythroblasts. This was supported by Patuleia and Friend (1967) who found that tissue cultures of undifferentiated reticulum cell sarcomas derived from spleen or liver cells of FV infected animals eventually matured into proerythroblasts. Boiron et al. (1965) suggested that the so-called "Friend Cell" is probably a proerythro-

blast. Later work by Reilly and Schloss (1971) suggested that the erythrocytes of infected animals can be carriers of FV particles. FV has been seen budding from megakaryocytes and plasma cells (Yumoto et al, 1965), erythroblasts, reticulum cells and even mature erythrocytes (Reilly and Schloss, 1971). Thomson (1969) postulated the myeloid series as the possible target cells of FV replication. This was based on the findings that the bone marrow, containing more myelocytes than lymphocytes was better able to transmit FV to lethally irradiated mice than was splenic tissue containing more lymphocytes than granulocytes. Bennett and Steeves (1970) suggest the target cells of FV replication are not undifferentiated stem cells but a cell between a stem cell and the more mature erythropoietin sensitive cell while Bainbridge and Bendinelli (1972) suggest the site of FV infection to be a hemapoietic stem cell.

Like all virus-induced leukemias, FV induced leukemia is immunodepressive in nature. One of the first immunosuppressive effects reported is a decrease in the ability of infected animals to respond to antigenic stimulation by sheep red blood cells (SRBC). The infected animals show reduced serum agglutinin levels and a decrease in the number

of plaque forming cells (PFC), (Morton and Siegel, 1966; Ceglowski and Friedman, 1969). Koo et al. (1971) related that the depression of antibody formation was evident to antigens as diverse as serum proteins, bacterial extracts and skin allografts. The degree of suppression is related to the time interval between infection and stimulation. The greatest suppression occurred when the animals are infected three to eight days prior to antigenic challenge. The degree of suppression depended on the virus dose (Koo et al., 1971). Bennett and Steeves (1970) showed that FV did not impair graft vs host (GVH) reactions, allogenic-sensitive reactions or the rejection of hemapoietic allografts, but FV did impair the host response to SRBC. They suggested that FV selectively affected precursors of humoral antibodies, the T lymphocyte population was not affected. However, it is now known that a B lymph - T lymph cooperation is necessary to produce a successful PFC response to SRBC (Brody, 1970). Work by Bendinelli (1971) showed that FV virtually abolished in mice the ability to produce PFC in response to SRBC.

Bainbridge and Bendinelli (1972) showed that FV infection greatly slowed the circulation of lymphoid cells in infected spleens. This would in turn affect any immune

response that necessitates lymphocyte cooperation. This was substantiated by a depressed SRBC response but a normal to enhanced allogenic lymphoid cell response. Evidence was presented that FV depressed the number of B cell precursors of the anti-SRBC response. Cerny and Halasa (1973) studied the number of antigen binding rosette forming cells (T lymphs) present in FV infected spleens. The study showed no inhibition of rosette formation by FV but an inhibition of the immune response to SRBC. It was suggested that the antigen reactive cells may remain preserved during a virus-induced leukemogenesis whereas cellular differentiation leading to antibody formation is inhibited. Work by Friedman and Ceglowski (1971) and Mortenson et al. (1973) showed that FV causes a marked depression in cell-mediated immunity as revealed by delayed-type hypersensitivity reactions. In vitro studies show a loss of the ability of the lymphocytes of the spleen to migrate. There is also the loss of the ability of such cells to produce a migration inhibition factor (MIF) as early as three days post-infection. The degree of loss was related to the time after infection and the dosage of FV. Macrophages were shown to still be able to respond to MIF but lymphocytes of FV-infected animals were not able to produce MIF.

Chemotherapy

Arabinosyl cytosine (Ara-C) is an analog of cytosine and was first synthesized in 1961 (Evans et al., 1961). Ara-C is a powerful inhibitor of DNA replication. The mode of action of the drug appears to be three-fold. It inhibits the reduction of the ribose diphosphate of cytosine to the corresponding deoxyribose derivative, Chu and Fischer (1962) and it also inhibits DNA polymerase (Kimball and Wilson, 1968; Graham and Whitman, 1970). Both of these events occur in the S-phase of the cell cycle. Ara-C also seems to have a limited affect on non-S-phase tumor cells. It seems to inhibit the incorporation of both DNA and RNA into polypeptides (Karon et al., 1972).

The drug exerts its effects for only a short period of time. It is rapidly inactivated in vivo by deamination to a uracil compound. The half-life in mouse serum for a dose of 50 mg/kg is about 37 minutes (Mulligan and Mellett, 1968). It is also excreted rapidly, up to 85% appears in the urine in a 24 hour period (Dixon and Adamson, 1965).

The brief active period coupled with the fact that Ara-C has its primary affect on cells passing through the S-phase reduces the effectiveness of Ara-C in eliminating

large numbers of tumor cells. This limitation can be partially overcome by the use of frequent, intermittent doses to act on each new segment of the cell population entering the vulnerable stage (Kline et al., 1966). A more effective solution is the synchronization of the entire cell population so that a large number of cells are in the S-phase at one time (Skipper et al., 1967). Such synchronization may be obtained by using a metaphase inhibitor such as vinblastine prior to administration of Ara-C (Valdamundi and Goldin, 1971).

Ara-C used against FV leukemia in BALB/c mice greatly lowers the number of erythroblasts and PMN's in the peripheral circulation (Chirigos, 1969). A regression in splenomegaly and a decrease in plasma virus titers is also observed (Chirigos et al., 1965). Ara-C seems to have little effect on the number of lymphocytes and monocytes. When the drug is discontinued, the depressed cell types reappear rapidly and overshoot normal numbers (Chirigos, 1969). It is not clear whether this is the result of direct antiviral action or whether it reflects a decrease in cell numbers capable of supporting virus multiplication.

Besides Ara-C cytotoxic actions against tumor cells, it is capable of inhibiting the replication of many viruses. Originally, only DNA and not RNA viruses were thought to be susceptible (Prince et al., 1969). However, evidence now shows that Moloney and Rauscher viruses, both containing RNA are prevented from multiplying by this drug (Hirschman et al., 1969). Since Rauscher virus is closely related to Friend virus, it is likely that Ara-C also inhibits its replication.

Ara-C like many antitumor agents has a suppressive affect on the host's immune system. Grimswold et al. (1972) found that Ara-C inhibits the 19's and 7's hemolysin plaque-forming cell responses. It also affected cell-mediated skin allograft responses. These effects depended on the dosage and time of administration. In mice, an early administration of Ara-C had no effect on skin allograft retention while a later dosage significantly prolonged the retention time. The lymphocytic cell line, for some unexplained reason, seems to be very susceptible to the toxic action of Ara-C (DiLorenzo et al., 1973). Work on Lin (1973) suggests that proliferating B lymphs are especially affected by the drug. Winkelstein (1973) found that the phytohemagglutin (PHA) response of T lymphs was impaired. The levels of MIF were

not reduced but a reduction in the number of macrophages was observed. He concluded that T lymphs as well as B lymphs were affected by Ara-C. In a study by Karchmer and Hirsch (1973), using Ara-C to treat zoster and herpes infections, a reduction in complement fixing antibody responses and interferon titers was also observed.

Vinblastine

Vinblastine is an alkaloid extracted from the periwinkle plant (Vinca rosea Linn.) and has the empirical formula $C_{46}H_{58}O_9N_4$. It previously had the generic name vincaleukoblastine abbreviated VLB. The substance was found to have antitumor activity and a toxicity low enough to permit it to be of clinical value (Johnson et al., 1960).

All aspects of the mechanisms of VLB action are not understood. VLB has conclusively been demonstrated to produce metaphase arrest (Cutts, 1961; Bruchovsky et al., 1965; Pfeiffer and Tolmach, 1967). Bensch and Malawista (1969) reported that VLB causes the microtubules of the spindle to "crystallize" in normal mammalian cells and thus their use in spindle construction is prevented.

Since the presence of cells to metaphase arrest does not seem to correlate with the therapeutic effects of VLB

(Svoboda, 1966), it is unlikely that this cytological affect is the only one the drug produces. Madoc-Jones and Mauro (1968) found that VLB causes death of HeLa cells during interphase as well as during mitosis. In other tissue culture studies, the interference with the metabolic pathways of amino acids leading from glutamic acid to the citric acid cycle and to urea is suggested (Johnson et al., 1960; Johnson et al., 1961). VLB may also inhibit RNA synthesis through its affect on the DNA-dependent RNA polymerase system (Creasey and Markin, 1964). A study by Plagemann (1970) suggests variable effects of VLB depending on its concentration. At low concentrations (0.01-0.1mM), metaphase arrest was observed with no effect on cell viability and RNA synthesis. At higher concentrations (0.2-0.6mM), there was inhibition of the incorporation of uridine into the nucleic acid pool, inhibition of the conversion of ribosomal precursor RNA into rRNA, dissolution of the nuclei, precipitation of intracellular material and subsequent cell death. Metaphase arrest was not observed.

VLB produces marked hematologic effects, the most pronounced is a leukopenia (Johnson et al., 1963). VLB affects both the myeloid and lymphoid cell series (Pfeiffer

and Tolmach, 1967). Large lymphocyte proliferation is inhibited which in turn limits an organism's ability to resist infection (McGregor and Logie, 1973).

VLB's primary time of action is during the mitosis phase of the cell cycle when it attacks rapidly-proliferating cells preferentially. Its effectiveness as an anti-cancer drug is probably related to this fact. Valeriote et al. (1965) found VLB to be more toxic to lymphoma cells than to normal hemopoietic cells in AKR/J mice. Likewise, they also observed that colony-forming cells of the spleen were more sensitive to VLB than those of the bone marrow.

Because of VLB's ability to produce metaphase arrest, it is a suitable agent for synchronizing a neoplastic cell population. When used against FV in combination with Ara-C, it should enhance the actions of the drug by rendering more cells in the S-phase of the cell cycle at one time. VLB in its own right should be beneficial because of its possible selectivity against tumor cells and its leucopenic effect.

Leucogenenol

Leucogenenol, a structurally complex molecule, with the properties of an enol and the molecular formula of $C_{18}H_{25}NO_8$ has been isolated from Penicillium gilmanii and

also human and bovine liver (Rice, 1966; Rice and Shaikh, 1970). Leucogenenol has been found to readily form salts with sodium, potassium, and calcium and these salt solutions probably retained their biological activity for over a year, however an aqueous solution of leucogenenol itself readily loses its activity at room temperature (Rice, 1971). When hydrolyzed leucogenenol forms the following compounds: 1,2 dihydroxy - 3-methyl-5-oxycyclohexanecarboxylic acid, 3-hydroxy-3-hydroxy-methyl-5-methylcyclohexane-1,2-dione, glycoaldehyde, amino-acetaldehyde and ammonia. This evidence and spectroscopic data indicates that leucogenenol is 2-(1,2-dihydroxy-3-methyl-5-oxocyclohexyl-3,11-dihydroxy - 11-(hydroxymethyl)-9-methyl-1-oxa-5-azaspiro 5,5 undeca-2,4-dien-7-one.

Injection of as little as 1 ug/kg of leucogenenol elicits a leucocytosis without a febrile response (Rice, 1968). It is not toxic in dosages as high as 500 ug/g in mice (Rice and Darden, 1968). Rice (1968) reported in mice (strain unspecified) that a large dose (200 ug/kg) of leucogenenol caused a decrease in neutrophils to one-half normal values and a corresponding greater than two-fold rise in lymphocytes. This was seen within four hours after injection

and persisted for twelve days. The total peripheral white blood cell count however remained relatively unchanged. Injection of leucogenenol either intravenously or intraperitoneally leads to an increase in the percent of neutrophils and later an increase in the percent of lymphocytes in the peripheral blood differentials of rabbits and mice (Rice et al., 1971a). Rice and Darden (1968) studied the effect of leucogenenol on bone marrow cells and found an increase in myeloblasts after 24 hours followed by a return to normal levels in five days. A slight increase in the nucleated erythroid cells was also observed. The basophilic, eosinophilic and megakaryocytic series of cells did not seem to be affected by leucogenenol. Leucogenenol given to sublethally irradiated mice resulted in an increase in peripheral blood lymphocytes and a two-fold increase in lymphoblasts in the spleen at 24, 48 and 120 hours after injection (Rice et al., 1968). A later study on irradiated mice given leucogenenol and SRBC indicated a shorter latent period before antibody production and greater hemolysin titers than were found in mice not given leucogenenol. They suggested that leucogenenol increases the rate of transformation of precursor cells into cells capable of antibody synthesis (Rice et al., 1970b).

The addition of leucogenenol to tissue cultures of human lymphoblastic cells increases their respiratory quotient and rate of cell division (Rice and McCurdy, 1972). Leucogenenol also affects the enzyme systems that are associated with lymphoblastic cell transformation (Rice and McCurdy, 1971).

Rice and Ciavarra (1971) and Rice et al. (1972) showed that leucogenenol stimulates the maturation rate of antibody producing cells and the subsequent elicitation of normal antibody titers in splenectomized rats. Autoradiographic studies of the action of leucogenenol using tritiated thymidine showed that the increase in PMN and the subsequent decrease 12 hours after injection was caused by an efficient sequestering mechanism in the rat. This proposal was supported by increased accumulation of labeled neutrophils in the spleen. The lymphocytosis occurring after injection was accompanied by an increase in labeled lymphs indicating an increased maturation of lymphoid precursors (Rice et al., 1971a). Increased label was found in the proliferating cells of the bone marrow, transformed cells of the myeloid series and lymphocytes of the spleen. Also, an increase was found in labeled prorubricytes indicating a stimulation of the maturation of at least one type of

nucleated erythroid cell (Rice et al., 1971b). The biological activity of leucogenenol seems to be to stimulate DNA replication in myeloid, lymphoid and some erythroid cells and increase their rate of transformation. Since leucogenenol effects at least three cell lines, it is probable that its stimulatory affect is on a primitive stem cell common to all three cell lines.

Rice et al. (1970a) and Rice and McCurdy (1973) suggest that leucogenenol is probably found in all mammalian tissues. They cite the liver, spleen, adrenals, gonads, and blood as examples. Its primary biological activity is that of regulation of the number and types of blood cells and thus their associated biological activities.

Four previous studies at Drake University have dealt with leucogenenol by itself and in conjunction with various methods of chemo- and immunotherapy in the treatment of FV leukemia and leukemia L1210. A study by Elliott et al. (1973) documents the enhancement of FV leukemia by leucogenenol. Significant elevations in the peripheral white blood cell counts were observed, also in the lymphocyte portion of the peripheral blood cell differentials and spleen and liver weight to total body weight ratios. A decrease in

survival times and an increase in hemagglutination and complement fixation titers to SRBC were observed when comparing FV + leucogenenol to FV infected animals. It was suggested that leucogenenol potentiated the leukemia by providing more sites in which the virus could replicate and more cells that could be transformed by it. Also, leucogenenol evidently counteracted some of the immunosuppressive effects of FV by producing near normal antibody titers in the infected animals.

Further studies by Averbeck (1973) and Muckerheide (1973) using leucogenenol and various immuno- and chemotherapeutic procedures showed that while the drugs themselves were effective in prolonging the lifespan and reducing the parameters of the FV leukemia, the addition of leucogenenol did not significantly enhance the drug's action. A later study of Barnhill (1973)using leucogenenol and chemotherapy in the treatment of leukemia L1210 showed mixed results. Although the lymphoid percent of the peripheral blood cell differentials, hematocrits and in some cases the antibody titers were increased, thereby showing beneficial effects, the survival times were not significantly increased and in the case of the addition of leucogenenol to chemotherapy were actually lowered. All studies of leucogenenol indicate that

it stimulates the humoral immune response in immuno-suppressed animals (Rice et al., 1968; Rice and Ciavarra, 1971; Elliott et al., 1973; Barnhill, 1973). The effects of leucogenenol on leukemias leading to enhancement suggest a stimulation of the cells dealing in humoral antibody production (B lymphs) and/or a suppression of the cell mediated immune response (T lymphs).

Lymphocytes

It is now known that the seemingly homogeneous lymphocyte fraction of the blood carries on at least two separate and distinct functions. Within the last ten years, these lymphocytes have been separated into two distinct groups on the basis of physical and chemical properties, immune reactions and cell membrane antigens. These lymphocytes are basically of the thymus (T) type concerned with cell-mediated immune reactions or Bursa equivalent (B) type concerned with humoral antibody production.

The need for quantification of the B or T lymphocytes has become increasingly apparent for at least four reasons. First, the recognition by Hellstrom et al. (1968) and others, that one of the main functions of T lymphs is the inhibition of growth or the destruction of tumor cells. Second, the

realization by Hellstrom and Hellstrom (1970), Hellstrom et al. (1971) and Sinkovics et al. (1972) that humoral antibodies may cause a phenomenon known as immunological enhancement of a tumor. It was found that humoral antibodies (called blocking antibodies) will bind to tumor cells and inhibit the action of T lymphs by making the tumor cells inaccessible to the cytotoxic action of T cells. Thirdly, work by Aisenberg and Block (1972) and Borella and Sen (1973) show that various human lympho-proliferating diseases are of predominately B or T lymph origin. Similar results have also been found in test animals. And finally, there is the realization of the immunosuppressive effects of cancer chemotherapeutic agents and their potential inhibition of the host's immune surveillance mechanisms (Penn and Starzi, 1973).

The lymphocytes dealing with cell mediated reactions are called T lymphocytes or thymus dependent lymphocytes. They have a theta (θ) antigen on the cell membrane that is also found on >95% of all thymus lymphocytes (Raff, 1969). The θ antigen has been used to develop cytotoxic assays, Shortman et al. (1972) and immunofluorescent assays Raff (1970) and Bankhurst and Warner (1971) for the quantification of T lymphs in mice. T lymphs will also spontaneously

form rosettes called E-rosettes with SRBC and this has also been used to quantitate them (Bianco and Nussenzweig, 1971). Reif and Allen (1964) showed a cross-reactivity of a θ antisera with mouse brain tissue. Golub (1971) used this information to make a rabbit anti-mouse brain-associated antisera (antiBA-θ) that after proper absorption, proved to be specific for T lymphs. His findings were later confirmed by Barker et al. (1973) and Hudson and Phillips (1973). T lymphs are largely responsible for the recognition and the cytotoxic affects of the host's immune system on tumor cells (Hellstrom and Hellstrom, 1970). Leclerc et al. (1973) showed that the thymus-processed lymphoid cell subpopulation showing the θ antigen is exclusively or predominantly responsible for the immune cytolysis both in a syngenic tumor system and in allogenic transplantation systems.

The lymphocytes dealing with humoral antibody responses are called B lymphocytes. B lymphs possess immunoglobulin determinants on their cell membranes, Raff et al. (1970), a receptor site for the third protein of complement (C3), Bianco and Nussenzweig (1971) and will form rosettes called EAC-rosettes with SRBC and an antibody to SRBC plus C3 (Jondal et al., 1972). Immunofluorescent techniques using anti-gammaglobulins have been developed for the detec-

tion and quantitation of B lymphs (Raff, 1970; Rabellino et al., 1971).

The purpose of this study was to quantitate the effects of leucogenenol and chemotherapy on the B and T lymphocyte populations in BALB/c mice infected with FV leukemia. The study attempts to answer the questions raised by previous studies at Drake University as to the specific immunocompetent lymphocyte populations stimulated or depressed by leucogenenol injections, FV infection and the combination of these.

MATERIALS AND METHODS

Mice

Female, BALB/c mice used throughout this study were obtained from the Jackson Laboratory, Bar Harbor, Maine or Cumberland View Farms, Clinton, Tennessee. All mice were from 12 to 24 weeks old. The mice were kept in disposable plastic cages in groups of five or six. Bedding was changed as needed and standard laboratory chow and water were given *ad libitum*.

Friend Virus

The strain of Friend virus used in these studies was obtained from the Drake University stock pool. The history of the virus has been previously described by Elliott et al. (1970). The virus was kept frozen at -70°C as 20% suspension of homogenized BALB/c mice spleens in sucrose stabilizer. The virus stock was removed from -70°C storage and thawed by swirling in a 37°C water bath. The suspension was transferred to a sterile centrifuge tube and spun in a refrigerated centrifuge at 4°C for 10 minutes at 2,000 x g. One ml of the supernatant fluid was drawn off and a 10^{-3} dilution made using sucrose stabilizer as the diluent. All mice given FV received 0.3 ml volume of the 10^{-3} virus suspension injected intraperitoneally (IP).

Leucogenenol

The calcium salt of leucogenenol was purchased from Pfanstiehl Laboratories, Inc., Waukegan, Illinois. When purchased, the salt had a biological activity rating of 50%. The salt was dissolved in sterile pyrogen-free distilled water to give a 50 ug/ml concentration. All mice receiving leucogenenol were given a 0.2 ml dose (10 ug) IP at 7 day intervals starting 3 days before virus infection.

Chemotherapy

Vinblastine sulfate (Velban, Eli Lilly and Co., Indianapolis, Indiana) was obtained as a 10 mg lyophilized plug. The plug was dissolved in sterile, pyrogen-free distilled water. A dosage of 1.08 mg/kg in a volume of 0.1 ml/mouse was injected IP.

Arabinosyl cytosine hydrochloride (Cytosar, The Upjohn Company, Kalamazoo, Michigan) was obtained in a lyophilized form and was dissolved in sterile, pyrogen-free distilled water at pH 7.0. A dosage of 20 mg/kg in a volume of 0.1 ml/mouse was injected IP. The drug sequence was given at 8 hour intervals on days 7 through 11 following virus infection. One dose of VLB was followed by 2 of Ara-C in a twenty-four hour period and then the sequence was repeated. Drug dosages were calculated on the basis of average mouse weights.

Preparation and Absorption of AntiBA-θ Serum

The antiBA-θ serum was prepared by the method of Golub (1971) and Barker et al. (1973). Five uninfected BALB/c mice were sacrificed by cervical dislocation and their brains aseptically removed under a laminar air flow hood. An equal volume of cold Hanks' Balanced Salt Solution (HBSS), (GIBCO), was added to the brains and then homogenized

in a Virtis homogenizer. To the homogenized brain suspension, an equal volume of Freund's complete adjuvant (DIFCO) was added and an emusion made. Emulsion volumes of 0.5 to 1.0 ml were injected subcutaneously at 2 sites on each of five rabbits. Each rabbit received a total of 1.5 ml. The procedure was repeated 7 days later. The rabbits were then bled by cardiac puncture on days 14 and 19. The serum, separated from the blood, was pooled and stored in 20 ml aliquots at -20°C.

The antiserum was absorbed using a procedure by Barker et al. (1973). A homogenate of BALB/c livers and erythrocytes was prepared in an equal volume of HBSS in a Virtis homogenizer. The liver-erythrocyte homogenate was washed 5 times with HBSS and 5 volumes of antisera were added to 1 volume of the homogenate. The mixture was incubated over ice for one hour with occasional shaking. After centrifugation, the antiserum was further absorbed with agarose (Nutritional Biochemicals Corp., Cleveland, Ohio) according to Cohen and Schlesinger (1970). To each ml of antiserum, 50 mg of agarose was added and the mixture incubated over ice for one hour with occasional shaking. After centrifugation and discarding the agarose, the antiserum was incubated

at 56°C for 30 minutes to inactivate complement and then stored in 1 ml aliquots at -20°C.

Complement Preparation

Rabbit complement (GIBCO) was obtained in a lyophilized form. After reconstitution, it was absorbed with a liver-spleen-erythrocyte homogenate by the procedure outlined by Barker et al. (1973) and further absorbed with agarose according to Cohen and Schleisinger (1970). To cut down on the loss of complement due to the swelling of agarose, the serum was diluted 1:3 with HBSS and the absorption was carried out using 80 mg of agarose per ml of complement. The absorbed complement was stored in 1 ml aliquots at -20°C.

Lymphocyte Isolation

The isolation procedure and subsequent assays were carried out in siliconized glassware. Each mouse was sacrificed by decapitation and its blood collected in aluminum weighing boats under a laminar air flow hood. Blood was immediately taken for nucleated cell counts and differentials before clotting occurred. The rest of the blood was added to an equal volume of Tris Balanced Salt Solution (Tris BSS) at pH 7.45 containing 10 units of heparin per ml. The

inguinal, brachial and axillary lymph nodes, the spleen and thymus were then removed and placed in vials of Tris BSS. These organs were ground into a fine suspension using a 10 ml glass syringe plunger. The mouse femurs were removed and the bone marrow forced out and collected using a 1 ml tuberculin syringe filled with Tris BSS and equipped with a 25 gauge needle.

The cell suspensions from the blood, bone marrow, lymph nodes, spleen and thymus were carefully layered over a solution made up 24 parts of 9% Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and 10 parts of 33% Hyapaque (Winthrop Laboratories, New York, N.Y.) in a 12 ml centrifuge tube. This solution had been made up prior to use with sterile pyrogen-free distilled water and had been warmed to 37^{o}C (Fotino et al., 1971). The tubes were then spun in a refrigerated centrifuge at 4^{o}C at 400 x g for 30 minutes. A beveled pasteur pipette was used to remove the lymphocyte rich layer and transfer it to 10 x 75 mm test tubes. These tubes were spun in a centrifuge at 2000 x g for 10 minutes. The excess media was removed and if erythrocytes were present, the cell pellet was resuspended in a tris-buffer-ammonium chloride solution pH 6.2-6.4 and allowed to stand one minute (Fotino et al., 1971). This lysed the erythrocytes and the

cell pellet was then washed 3 times in Tris BSS. Cell viability and concentration was determined using the trypan blue exclusion technique in a hemacytometer. All lymphocyte suspensions were finally adjusted to contain 2 x 10^6 viable cells per ml.

Cytotoxic Assay

The cytotoxic assay procedure was done by the method of Shortman et al. (1972). To 0.1 ml of each lymphocyte cell suspension, containing 2 x 10^6 cells, 0.1 ml of a suitable dilution of absorbed antiBA-θ was added and incubated at room temperature for 30 minutes. To this, 0.1 ml of 1:2 diluted and absorbed rabbit complement was added and the mixture was incubated at 37°C for 30 minutes. Then 0.1 ml of 0.16% trypan blue in Tris BSS was added and the cell suspension put on ice. The viability of the cell suspension was assayed by counting 200 cells in a hemacytometer. All dilutions and suspensions were added using Eppendorf Microliter pipettes. Cells destroyed by the antiBA-θ and complement showed up as blue cells and were designated as T lymphocytes. Complement, antiserum and thymus controls were run with each assay to monitor specificity. Greater than 90% cell death in a thymus cell suspension is considered a

positive test of serum specificity. Data was recorded as % positive cells using the following formula (Konda et al., 1973).

% positive cells =

$$\frac{\text{N positive in test - N positive in controls x 100}}{\text{N alive in controls}}$$

Fluorescent Assay

A fluorescein conjugated goat anti-mouse gamma-globulin (Cappel Laboratories, Inc., Downington, Pennsylvania) was used for a direct fluorescent antibody technique of identifying B-lymphocytes (Rabellino et al., 1971). To the cell pellet obtained after 0.2 ml of each lymphocyte cell suspension was centrifuged, 0.05 ml of 1:4 diluted fluorescent antisera was added and incubated at room temperature for 45 minutes. After the cells were washed 3 times in Tris BSS, the cells were resuspended in a mounting media made up of 9 parts glycerol and 1 part pH 7.2 phosphate buffered saline.

One drop of the cell suspension was placed on a slide, covered with a coverslip and sealed with nail polish. The slides were examined with a Zeiss Photomicroscope II under both ultraviolet and phase contrast. For fluorescence, the microscope was equipped with an Ossran HBD 200 mercury arc

lamp. A BG12 primary filter and a 44-50 barrier filter combination was used for examining 200 cells at 600X magnification. Lymphocytes showing fluorescence were considered B lymphocytes. Serum specificity was determined by thymus controls and trypsin treatment of the cell suspensions which results in a greatly decreased staining reaction. Since the immunoglobulin reactive sites are cell membrane bound, a partial reaction of the cell with trypsin removes some of the reactive sites and a decreased staining reaction is observed.

Experimental Outline

A total of 54 mice were analyzed as to B and T lymphocyte percentages. Extra mice were included in some groups to allow for study of survival times. The following groups of mice were set up and treated as indicated:

Group I: Untreated Controls

Group II: Leucogenenol Controls

Group III: FV Controls

Group IV: FV + Leucogenenol

Group V: FV + Leucogenenol + Ara-C + VLB

Three mice from each group were sacrificed at weekly intervals following the start of treatment. Groups were put on

staggered treatment schedules to allow for full attention of one group per day. At the time of sacrifice, blood for nucleated cell counts and nucleated cell differentials was taken. The nucleated cell counts were made with the Coulter Counter, Model B, in which 20 λ of blood was diluted in 10 ml of diluting fluid (Counter Counter, Inc., Hialeah, Florida). Differentials were stained with Wright's stain and 100 cells were counted and classified. Multiple counts were made on questionable differentials and the average of these counts used. Also spleen, liver and body weights were recorded to monitor disease progression. The group of mice receiving chemotherapy was analyzed at weeks 2 and 4 only.

Results were statistically analyzed by means of a standard t test. Values at the 90% ($p < 0.1$) confidence level or greater were considered significant.

RESULTS

The values for untreated and leucogenenol control animals were obtained throughout the course of the four week experiment but will be discussed as single values. All other comparisons, that is FV controls, FV + Leucogenenol, and FV + Leucogenenol + Ara-C + VLB groups are made on a weekly

basis and analyzed with a corresponding group of the same week. It was found in many cases that due to the small sample size of three animals per group that the statistical results gave slightly or questionable significant values ($p < .2$). It is felt in many of these cases that if larger sample sizes could have been used, the results would have been significant ($p < .1$).

The results of the peripheral nucleated cell counts and peripheral leucocyte differentials will not be presented individually. Rather, Table 1 and Figure 1 refer to average total peripheral lymphoid cell counts given in $x\ 10^3$ cells/cu mm. The total peripheral lymphoid cell count/cu mm was obtained by multiplying each animal's total peripheral nucleated cell counts in cells/cu mm by the percent of lymphoid cells found in its differential. To avoid confusion and controversy, the term lymphoid cell will be used instead of lymphocyte. Thus, small, large and immature lymphocytes were all classified in one category.

Upon comparing the leucogenenol control's average of 8.07×10^3 cells/cu mm with the normal animal's average of 3.99×10^3 cells/cu mm, a significant increase ($p < .001$) was found. Mice given FV + leucogenenol showed higher total

peripheral lymphoid cell counts at all four testing periods than the FV control animals. However, only at week two were the values statistically significant ($p < .1$), when the values obtained were 18.15×10^3 cells/cu mm for the FV + leucogenenol group and 5.27×10^3 cells/cu mm for the FV group. The FV control value of 5.27×10^3 cells/cu mm for the second week when compared with the FV + leucogenenol + Ara-C + VLB group value of 2.27×10^3 cells/cu mm had the same significance ($p < .1$) as did the fourth week values of 59.85×10^3 cells/cu mm and 24.15×10^3 cells/cu mm respectively. Comparisons of the total peripheral cell counts between the FV + leucogenenol group and the FV + leucogenenol + Ara-C + VLB group for the second and fourth week were also significant ($p < .1$) with values of 18.15×10^3 cells/cu mm to 2.27×10^3 cells/cu mm and 74.36×10^3 cells/cu mm to 24.15×10^3 cells/cu mm.

The simple percentage values for B and T lymphocyte assays in the peripheral blood are not presented. Instead, these results are multiplied by the total peripheral lymphoid cell counts to give the absolute number of peripheral B and T lymphoid cell counts $\times 10^3$ cells/cu mm. The B and T lymphocyte results are combined in tables and charts for ease of interpretation.

Table 1. Average Total Peripheral Lymphoid Cell Counts x 10^3 Cells/cu mm.

Animal Group	Week	Total Lymphoid Cell Count x 10^3 cells/cu mm	SD
Group I Normal Controls	1	3.66	1.06
	2	3.82	.45
	3	4.48	.34
	4	3.99	.72
Group II Leucogenenol Controls	1	7.70	.71
	2	7.71	.61
	3	7.36	.33
	4	9.49	3.13
Group III FV Controls	1	5.13	.86
	2	5.27	1.44
	3	51.94	8.28
	4	59.85	25.78
Group IV FV + Leucogenenol	1	5.89	.61
	2	18.15	9.42
	3	73.96	38.07
	4	74.36	32.50
Group V FV + Leucogenenol + Ara-C + VLB	2	2.27	.97
	4	24.15	3.03

SD = standard deviation in 10^3 cells/cu mm.

Figure 1. Total peripheral lymphoid cell counts x 10^3 cells/cu mm. All values are the average of 3 animals.

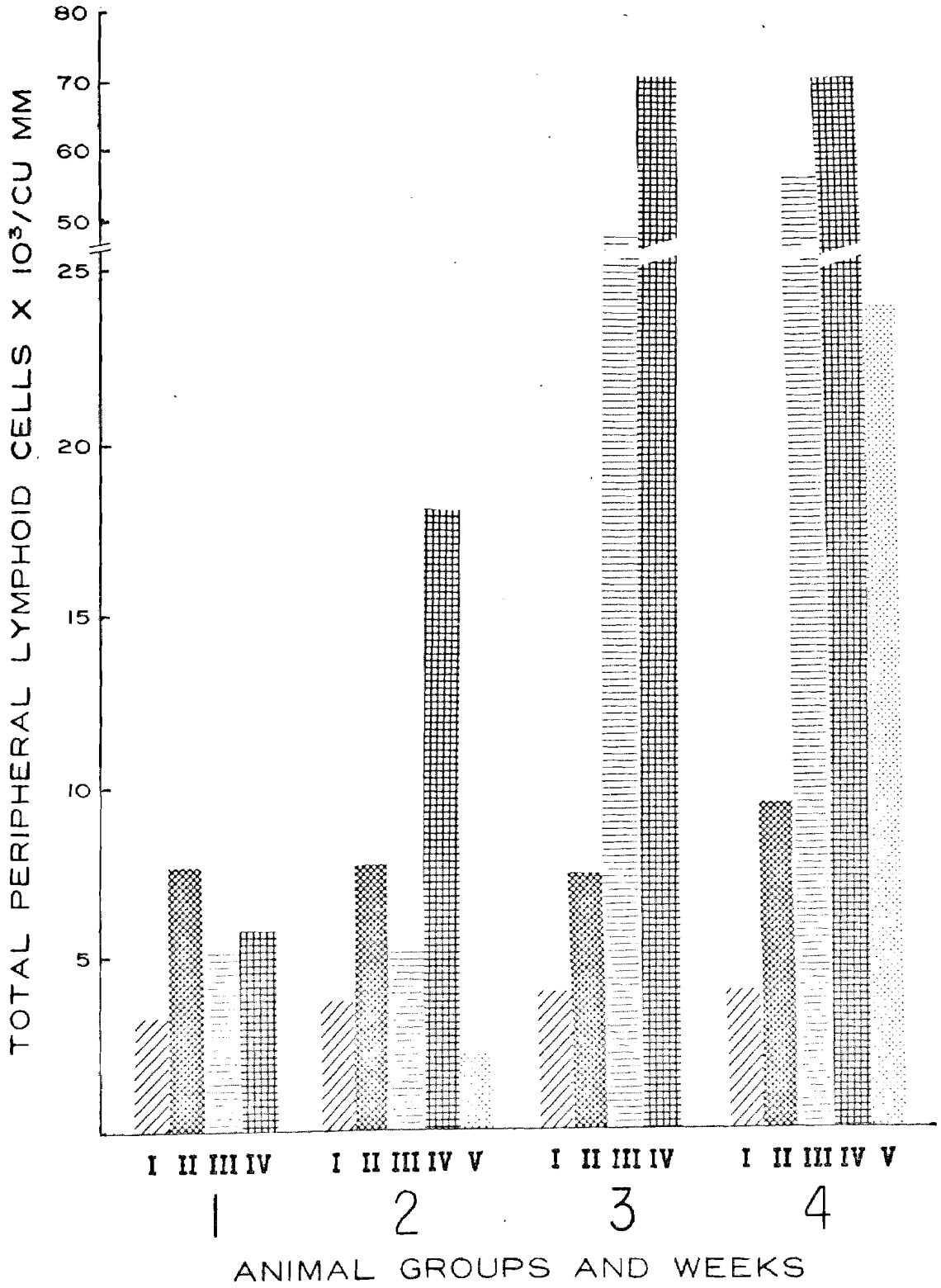
Group I: Normal Controls

Group II; Leucogenenol Controls

Group III: FV Controls

Group IV: FV + Leucogenenol

Group V: FV + Leucogenenol + Ara-C + VLB


TOTAL PERIPHERAL LYMPHOID CELLS X 10^3/CU MM
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60
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I II III IV
I II III IV V
I II III IV
I II III IV V
1
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ANIMAL GROUPS AND WEEKS

Table 2 and Figure 2 refer to the average absolute peripheral B and T lymphoid cell counts x 10^3 cells/cu mm in the peripheral blood. The B lymph population average of 3.66 x 10^3 cells/cu mm in the leucogenenol control group is a significant increase ($p < .001$) over the normal animal average of 1.79 x 10^3 cells/cu mm. No significant difference was found in the B lymph population of the FV controls and FV + leucogenenol groups at weeks 1, 3 and 4. However, the second week B lymph value of 6.47 x 10^3 cells/cu mm for the FV + leucogenenol group when compared with FV control value 3.38 x 10^3 cells/cu mm was slightly significant ($p < .2$). The FV controls when compared with the FV + leucogenenol + Ara-C + VLB animal group at week 2 had a significant elevation ($p < .05$) of B lymphs with values of 3.38 x 10^3 and 1.02 x 10^3 cells/cu mm respectively. By week 4 the values of 47.25 x 10^3 and 11.51 x 10^3 cells/cu mm were even more significant ($p < .01$). In comparing FV + leucogenenol group animals with FV + leucogenenol + Ara-C + VLB group animals, significant decreases in the B lymph values for the drug treated animals at weeks 2 and 4 were found. The B lymph values of 6.47 x 10^3 and 1.02 x 10^3 cells/cu mm at week 2 had a significance of ($p < .05$). The fourth week values of

39.65 x 10^3 and 11.51 x 10^3 cells/cu mm were significant at the ($p < .1$) level.

The T lymph values of 1.84 x 10^3 cells/cu mm in the leucogenenol controls is significant ($p < .01$) when compared to the average of .83 x 10^3 cells/cu mm in the untreated animals. No significant difference was found in the T lymph values of the FV controls and FV + leucogenenol results of the first week but the second week values of .67 x 10^3 cells/cu mm versus 2.57 x 10^3 cells/cu mm was significantly higher ($p < .1$). The third and fourth week values were still elevated in the FV + leucogenenol group but only slightly significant ($p < .2$), 9.95 x 10^3 versus 19.9 x 10^3 cells/cu mm and 8.94 x 10^3 versus 12.14 x 10^3 cells/cu mm respectively. At week two the T lymph values for the FV controls when compared with the FV + leucogenenol + Ara-C + VLB animal's group showed a decrease, but not a significant one, while the fourth week results of 8.94 x 10^3 cells/cu mm and 3.04 x 10^3 cells/cu mm were slightly significant ($p < .2$). In comparing FV + leucogenenol animals with FV + leucogenenol + Ara-C + VLB group animals, significant results in the T lymph values at weeks 2 and 4 were found. The second week values of 2.57 x 10^3 versus .55 x 10^3 cells/cu mm showed a significant

decrease ($p < .05$) while the fourth week values were 12.14 x 10^3 as opposed to 3.04 x 10^3 cells/cu mm ($p < .1$).

Table 3 and Figure 3 refer to the percent B and T lymphoid cells found in spleen preparations. No significant change was found in the B lymph percentages when comparing normals and leucogenenol controls. A consistent and significant decrease in the percent of B lymphs between the FV controls and FV + leucogenenol animals was found. Thus, second week values of 64.7% compared to 41.8% ($p < .05$) was found while third week values were 69.9% as compared to 42.2% ($p < .02$) and finally for the fourth week values of 59.6% and 46.2% ($p < .02$) was found. The comparison of the FV controls and FV + leucogenenol + Ara-C + VLB animals at weeks 2 and 4 for B lymphs reveals 64.7% and 54.6% as compared to 59.6% and 49.7%. Both sets of values are slightly significant ($p < .2$). In comparing the FV + leucogenenol group with the FV + leucogenenol + Ara-C + VLB animals, an increase in both the B and T lymph percentages was observed, however, only the B lymph values of 41.8% as compared to 54.6% at week 2 were significant ($p < .01$).

A significant decrease ($p < .1$) in T lymphs in the spleen preparations was found between the leucogenenol

Table 2. Average Total Peripheral B and T Lymphoid Cell Counts x 10^3 Cells/cu mm.

Animal Group	Week	Average Total Peripheral B Lymph Cell Count	SD	Average Total Peripheral T Lymph Cell Count	SD
Group I Normal Controls	1	1.49	0.19	.82	.63
	2	1.64	.58	.75	.09
	3	2.25	.22	.97	.18
	4	1.79	.48	.83	.30
Group II Leucogenenol Controls	1	3.76	.76	1.53	.20
	2	3.35	.22	1.43	.84
	3	3.36	.50	1.76	.75
	4	4.15	.91	2.64	.69
Group III FV Controls	1	2.37	.23	.56	.11
	2	3.38	1.08	.67	.12
	3	44.10	6.61	9.95	3.11
	4	47.25	8.33	8.94	5.18
Group IV FV + Leucogenenol	1	2.76	.40	.78	.40
	2	6.47	2.23	2.57	1.00
	3	49.56	27.73	19.91	8.72
	4	39.65	18.14	12.14	5.59
Group V FV + Leucogenenol + Ara-C + VLB	2	1.02	.40	.55	.39
	4	11.51	.61	3.04	1.28

SD = standard deviation x 10^3 cells/cu mm

Figure 2. Total peripheral B and T lymphoid cell counts x 10^3 cells/cu mm. All values are the average of 3 animals.

Group I: Normal Controls

Group II: Leucogenenol Controls

Group III: FV Controls

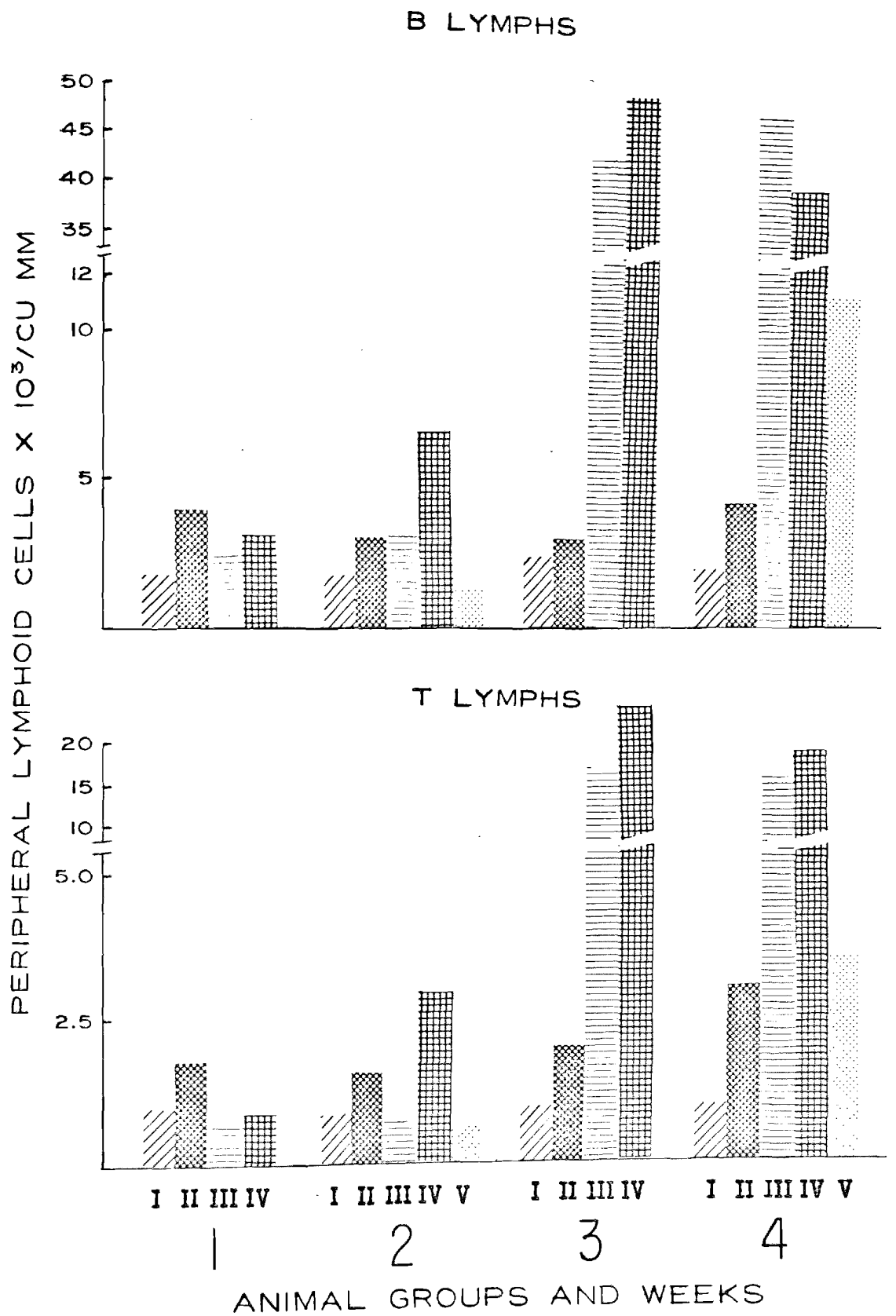
Group IV: FV + Leucogenenol

Group V: FV + Leucogenenol + Ara-C + VLB

phoid

the

VLB



control value of 51.0% and the untreated animal value of 44.4%. The T lymph values for the FV controls of 32.7% and 25.2% for the first and second weeks when compared with the FV + leucogenenol values of 17.7% and 9.1% respectively were reduced ($p < .2$). It was impossible to compare weeks 3 and 4 due to the inability to find any T lymphs in the FV + leucogenenol animals at that time. The second week FV controls and FV + leucogenenol + Ara-C + VLB T lymph values of 25.2% and 11.6% were slightly significant ($p < .2$).

Table 4 and Figure 4 refer to the average percent B and T lymphoid cells of the lymph nodes. In comparing the leucogenenol controls and the untreated animals, the average values of 47.9% and 38.7% for B lymphs proved to be slightly significant ($p < .2$). The FV controls and FV + leucogenenol controls showed significant decreases in the B lymph percentages at week two, values of 57.1% as compared to 44.8% ($p < .01$) and week three 61.2% as compared to 34.4% ($p < .05$). In comparing the FV controls with the FV + leucogenenol + Ara-C + VLB animals, no significant change was observed in the B lymph percentages. The FV + leucogenenol group 44.8% compared with the FV + leucogenenol + Ara-C + VLB group 53.8% showed a significant increase in the second week B lymph values ($p < .1$).

Table 3. Average Percent B and T Lymphoid Cells in Spleen Preparations.

Animal Group	Week	Average % B Lymphs	SD	Average % T Lymphs	SD
Group I Normal Controls	1	45.9	3.76	48.1	4.52
	2	49.8	1.57	32.2	14.83
	3	50.0	2.81	52.9	6.73
	4	48.6	3.30	44.4	18.60
Group II Leucogenenol Controls	1	48.8	.70	28.9	3.71
	2	55.8	3.69	30.3	9.68
	3	46.1	7.84	30.5	6.54
	4	53.3	2.69	11.9	6.48
Group III FV Controls	1	53.9	10.82	32.7	5.54
	2	64.7	9.12	25.2	12.40
	3	69.9	8.37	4.9	6.95
	4	59.6	7.09	11.6	16.35
Group IV FV + Leucogenenol	1	52.2	2.62	17.7	12.92
	2	41.8	2.00	9.1	7.16
	3	42.2	.61	0.0	---
	4	46.2	5.49	0.0	---
Group V FV + Leucogenenol + Ara-C + VLB	2	54.6	2.87	11.6	3.41
	4	49.7	6.26	0.0	---

SD = standard deviation in %

Figure 3. Percent B and T lymphoid cells in spleen preparations. All values are the average of 3 animals.

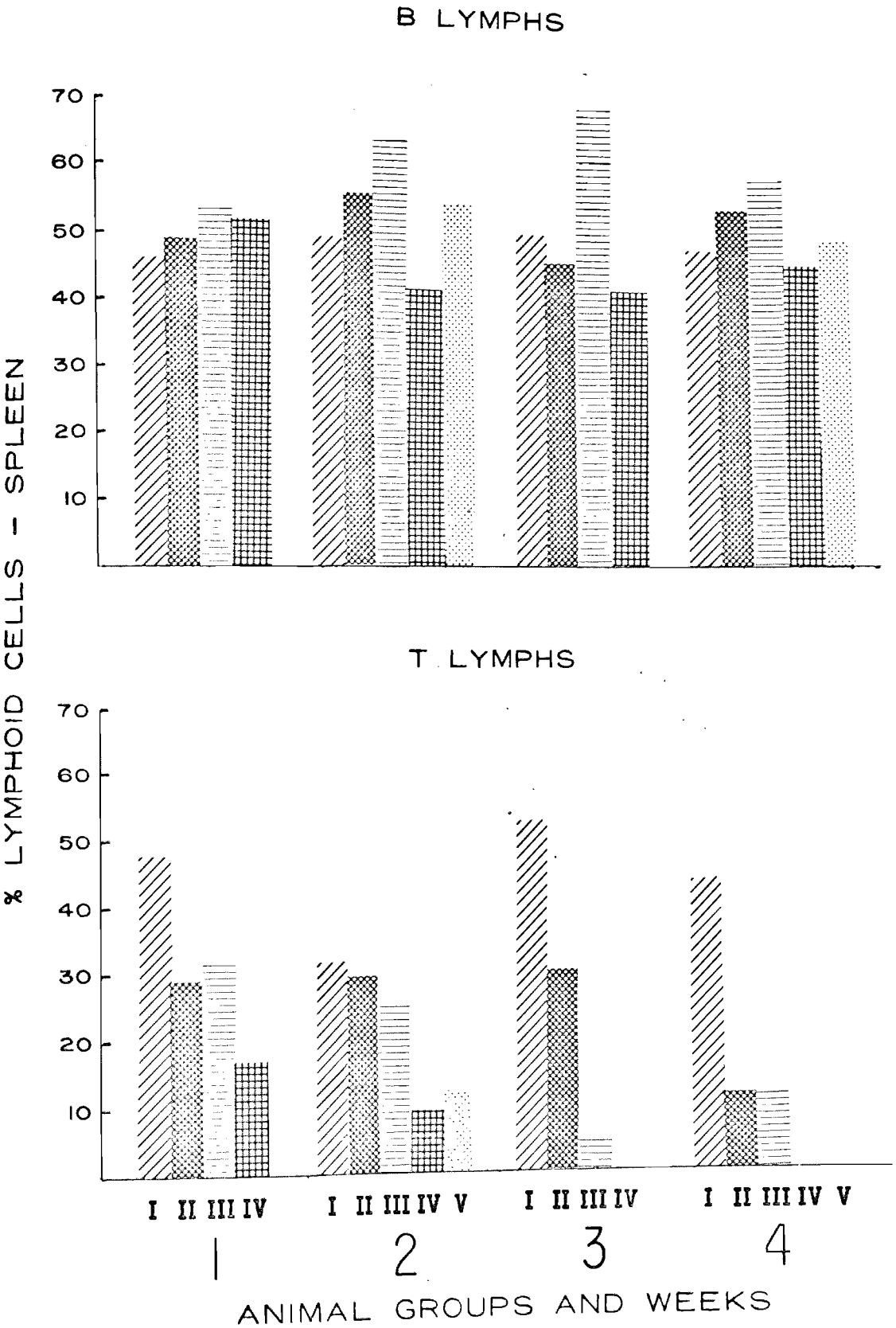
Group I: Normal Controls

Group II: Leucogenenol Controls

Group III: FV Controls

Group IV: FV + Leucogenenol

Group V: FV + Leucogenenol + Ara-C + VLB


B LYMPHS
T LYMPHS
% LYMPHOID CELLS - SPLEEN
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ANIMAL GROUPS AND WEEKS

The T lymph values for the normals and leucogenenol controls showed a significant ($p < .1$) reduction from 63.8% to 47.6%. The FV control and FV + leucogenenol values for the T lymph percentages were not significant at any testing period. A significant decrease ($p < .05$) was found in the fourth week values of 41.7% for the FV controls as compared to 11.7% for the drug group. The FV + leucogenenol group compared with the FV + leucogenenol + Ara-C + VLB group showed a significant decrease in the T lymph values at week two, 44.9% and 30.9% ($p < .02$), and the fourth week values of 52.3% and 11.7% ($p < .001$).

Table 5 refers to the average percent B and T lymphoid cells of the bone marrow. No consistent, significant change was found in making comparisons between any of the groups.

Table 6 refers to the average ratio of the combined spleen and liver weights to total body weight. No significant difference was found between the normal and leucogenenol controls. Although consistent increase was observed in the FV + leucogenenol controls when compared with FV controls, the increase never proved to be statistically significant. The FV controls when compared with the FV + leucogenenol + Ara-C + VLB groups showed average values of .092 and .084

Figure 4. Percent B and T lymphoid cells in the lymph nodes. All values are the average of 3 animals.

Group I: Normal Controls

Group II: Leucogenenol Controls

Group III: FV Controls

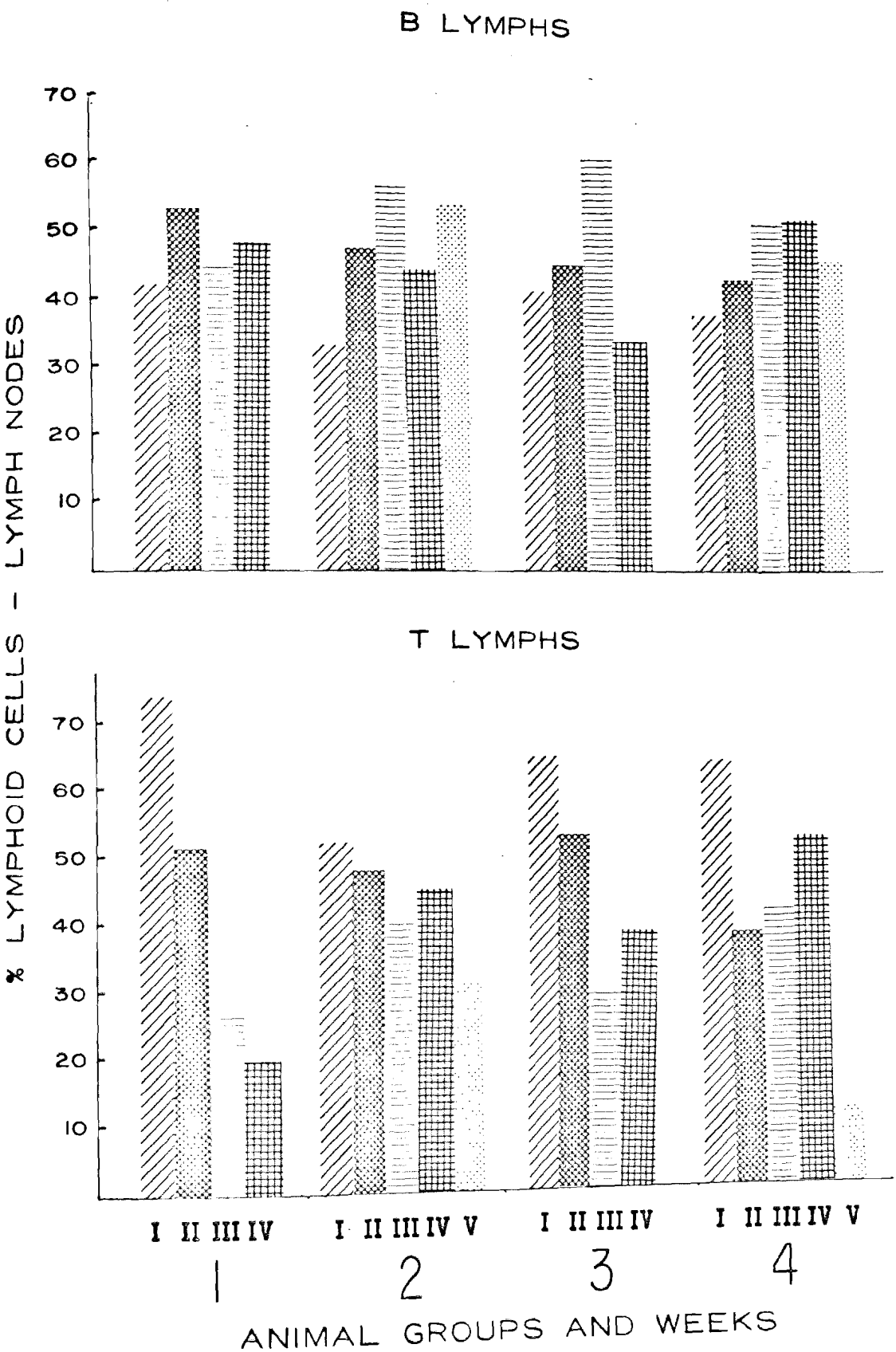
Group IV: FV + Leucogenenol

Group V: FV + Leucogenenol + Ara-C + VLB

Table 4. Average Percent B and T Lymphoid Cells in Lymph Nodes.

Animal Group	Week	Average % B Lymphs	SD	Average % T Lymphs	SD
Group I Normal Controls	1	41.9	8.52	74.3	11.95
	2	33.0	4.94	52.6	14.57
	3	41.3	3.85	64.4	13.30
	4	38.7	10.18	63.8	15.04
Group II Leucogenenol Controls	1	53.3	5.44	52.3	9.69
	2	48.1	14.42	47.6	7.10
	3	46.0	10.52	53.0	5.73
	4	44.1	11.19	37.6	5.19
Group III FV Controls	1	44.7	6.96	26.8	1.80
	2	57.1	2.23	39.5	13.50
	3	61.2	7.53	29.8	6.29
	4	51.8	5.80	41.7	11.69
Group IV FV + Leucogenenol	1	48.5	3.35	19.7	9.96
	2	44.8	2.22	44.9	2.90
	3	34.4	.70	37.4	7.40
	4	53.1	7.84	52.3	2.17
Group V FV + Leucogenenol + Ara-C + VLB	2	53.8	5.79	30.9	1.48
	4	47.7	5.70	11.7	.52

SD = standard deviation in %


B LYMPHS
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% LYMPHOID CELLS - LYMPH NODES
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ANIMAL GROUPS AND WEEKS

Table 5. Average Percent Lymphoid Cells in Bone Marrow.

Animal Group	Week	Average % B Lymphs	SD	Average % T Lymphs	SD
Group I Normal Controls	1	42.1	6.84	6.8	9.15
	2	40.9	3.43	5.8	8.20
	3	43.0	3.95	4.1	3.04
	4	42.0	4.99	5.6	7.35
Group II Leucogenenol Controls	1	48.7	4.70	5.2	4.86
	2	37.9	3.29	2.2	3.65
	3	47.7	4.71	6.8	2.70
	4	45.9	5.29	6.1	3.37
Group III FV Controls	1	53.6	5.72	3.8	5.42
	2	43.1	6.77	1.2	.92
	3	64.8	4.27	5.7	6.44
	4	42.9	5.96	4.3	6.13
Group IV FV + Leucogenenol	1	42.3	2.90	7.0	2.58
	2	47.8	3.56	6.0	2.20
	3	44.2	1.47	5.9	3.30
	4	46.2	3.23	8.8	7.35
Group V FV + Leucogenenol + Ara-C + VLB	2	46.9	2.53	5.5	2.09
	4	49.0	7.77	1.6	2.22

SD = standard deviation in %

Table 6. Average Ratio of the Combined Spleen and Liver Weights to Total Body Weight.

Animal Group	Week	Combined Liver + Spleen wt./body wt. (Average)	SD
Group I Normal Controls	1	.042	.00089
	2	.037	.00032
	3	.043	.00411
	4	.041	.00383
Group II Leucogenenol Controls	1	.039	.00965
	2	.038	.00259
	3	.041	.00023
	4	.051	.00413
Group III FV Controls	1	.055	.00603
	2	.092	.00595
	3	.158	.01139
	4	.200	.01661
Group IV FV + Leucogenenol	1	.045	.00457
	2	.101	.01723
	3	.160	.02530
	4	.211	.04452
Group V FV + Leucogenenol + Ara-C + VLB	2	.084	.00473
	4	.134	.00521

SD = standard deviation

respectively at week 2 ($p < .2$). The fourth week values of .200 and .134 for the same groups were significant ($p < .05$). The FV + leucogenenol animals being compared with the FV + leucogenenol + Ara-C + VLB animals for the second week showed values of .101 as compared to .084 ($p < .2$) and fourth week values of .211 as compared to .134 ($p < .050$).

In comparing the FV group with the FV + leucogenenol group, a slight decrease in survival times and an increase in spleen and liver weight to body weight ratios was observed, however the differences in both cases were not significant.

DISCUSSION

The BALB/c mice T lymphocyte values of 20.4% for peripheral blood, 44.4% for the spleen, 63.8% for the lymph nodes and 5.2% for the bone marrow correspond closely to the values of 25%, 40%, 62% and 3% respectively found by Stobo and Paul in 1973. These T lymph values coupled with the specificity testing using a percent thymus control death of greater than ninety percent also corresponds to the results found by Golub (1971) and Konda et al. (1973) for the use of the complement-associated cytotoxic assay technique utilizing antiBA-θ antiserum.

The B lymph values of 45% for the peripheral blood, 48.6% for the spleen, 38.7% for the lymph nodes differ somewhat from the 25%, 50% and 25% values for cell bound immunoglobulin containing cells or B lymphs as reported by Unanue et al. (1973). Other reports by Rabellino et al. (1971) and Jones et al. (1971) give more widely varying results. Rabellino reported values of 45% for the spleen and bone marrow and 14% for the peripheral blood and lymph nodes while Jones reports values of 34% for the blood, 29% for the spleen and 18% for the lymph nodes. The discrepancies in the results can be postulated to have come from various sources including the preparation of the antisera used, the cell isolation and testing assays involved, the age, sex, strain, or health of the mice and the mechanics of the operations involved. Wide variations in the percentages of B and T lymphocytes for various mouse strains have been reported. Reports by Raff et al. (1970); Golub (1971); Shortman et al. (1972); Hudson and Phillips (1973) and Unanue et al. (1973) all give widely varying lymphocyte population percentages and could be due to any of the above reasons.

The area of B and T lymphocyte identification and quantitation is still new and many conflicting and contradictory reports can be found. Lamelin et al (1972) stated

that cell-bound immunoglobulins are found on B lymphs and not on T lymphs but that not all B lymphs can be identified in such a manner. Stobo et al. (1973a) suggest a division of the T lymph population into at least two subpopulations based on the concentration of the θ-antigen, recirculating ability, radiation sensitivity, location of the cells and differences in cell mediated responses. Stobo et al. (1973b) also identified a lymphocyte population in BALB/c mice that has no θ antigen, no cell-bound immunoglobulin and no receptor for the third unit of complement. This population makes up an average of 10.3% of the spleen and 6.4% of the lymph node lymphocytes. Finally, Dickler et al. (1974) gave evidence for a population of lymphocytes between 1 and 6% that contain both a θ antigen as well as a cell-bound immunoglobulin.

The effects of leucogenenol on the B and T lymphocyte populations when compared with the normal mouse lymphocyte populations were as follows: a significant increase in the number of both B and T labeled lymphocytes in the peripheral blood, a slight increase in the percent of B lymphs in both the spleen and lymph nodes and a decrease in the percent of T lymphs in both organs. Since the only real quantitative

results were obtained from the blood, these values are indicative of the probable quantitative changes that took place in the spleen and lymph nodes. These organs are the source of most if not all of the circulating lymphocytes in the adult animal. The observed slight increase in the spleen and liver weight to total body weight ratios could be construed as due to the gross blastic stimulating affect of leucogenenol. Work by Elliott et al. (1973) indicates a significant increase in peripheral nucleated cell counts of leucogenenol animals as compared to normals. Rice (1968) and Rice et al. (1968) showed a two-fold increase in lymphocytes and in the relative number of lymphoblast cells. The results here also show a two-fold increase in peripheral lymphocytes. These results are suggestive of leucogenenol's probable role as a normal hemopoietic cell growth stimulator as suggested by Rice et al. (1970a) and Rice et al. (1973). The suggestion by Elliott et al. (1973) of leucogenenol acting as a stimulator of hemopoietic stem cells and lymphoblasts and the significant quantitative increase in the number of both B and T lymphs of the peripheral blood found in this study indicate that a preferential stimulation of B or T lymph stem cells is probably not true but rather a stimulation of both stem cell lines is indicated.

The enhancing effect of leucogenenol on FV leukemia can also be shown from the data. The significant increase in total peripheral nucleated cell counts in the second week indicates a more rapid progression of the leukemia. Friend virus's ability to replicate in progenitors of B lymphs was shown by Bennett and Steeves in 1970. Since the leucogenenol controls showed a significant increase in blood B and T lymphs over normal mice, stimulation of these hemopoietic stem cells must have occurred. The increase in the number of lymphoid stem cells means an increase in the number of cells suitable for FV replication. Therefore, a potentiation of the disease as observed by Elliott et al. (1973) could occur. The increase in virus replication would reduce the number of B and T lymphs that would reach maturation in the spleen and therefore a lowering of the percent of labeled B and T lymphs would be observed, since these lymphocytes must mature to be detected by the methods used in these experiments. The increase in the number of peripheral B lymphs in FV infected mice given leucogenenol would indicate an increase in cells capable of antibody production. This was shown to be true by Elliott et al. (1973). This antibody production leading to normal humoral antibody titers in FV immunosuppressed mice

could produce tumor enhancement through the production of blocking antibodies. Sinkovics et al. (1972) reported that the attachment of humoral antibodies to tumor cells leads to inhibition of the cytotoxic effects of T lymphs, thereby enhancing tumor growth.

Other immunosuppressive effects of the FV could counteract the increase in T lymphs brought about by leucogenenol in at least two ways. Bainbridge and Bendinelli in 1972 showed that the transit of lymphoid cells in an infected spleen is greatly slowed, therefore any reaction involving lymphocyte cooperation would be impeded. Mortenson et al. (1973) reported that as early as three days post-FV-infection, the ability of lymphocytes to produce MIF was lost. This would in turn mean that a successful T lymphocyte cooperative immune surveillance reaction responsible for controlling the emergence of a neoplasia would be reduced or eliminated in the FV infected mice (Mortenson et al., 1973). Therefore the increase in peripheral T lymphocytes found in these experiments could be negated by the impeded circulation of lymphocytes through the spleen and the loss of the T lymphocyte ability to produce MIF and mount a successful immune surveillance reaction.

The inability to find T lymphs in the spleen of FV + leucogenenol infected animals and FV + leucogenenol + Ara-C + VLB animals in the final stages of the disease is probably due to the dilution of immunocompetent cells (Dracott et al., 1972). Since Rice et al. (1972) showed an increase in the rate of transformation of immunoincompetent cells into active antigen reactive lymphocytes, the significant decrease in the percent of B lymphs in the lymph nodes might be explained by a shortened time of maturation and release leading to a reduced buildup of mature immunoglobulin cell-bound lymphocytes in the lymph nodes.

The effects of the drugs, Ara-C and VLB was to significantly lower the total lymphoid cell counts and to significantly decrease the identifiable B and T lymphs in the peripheral blood. These decreases would tend to suggest a retardation of the FV leukemia through the lowering of the lymphoid cell numbers. The reduced B lymph percentages in the spleen and lymph nodes would also lend support to the retarding effect of the drugs through a reduction in the number of cells suitable for FV replication. Animals given FV + leucogenenol when compared to the drug group gave less significant lymphoid cell counts and percentages of identi-

fiable B or T lymphs in the peripheral blood. It is likely that the drug's cytotoxic effect on the tumor cells was counteracted by the stimulatory affects of the leucogenenol. Ara-C has been shown by Grimswold et al. (1972) to inhibit hemolysin production and prolong graft retention which means that Ara-C effects both B and T lymphocyte responses. Lin in 1973 showed that combined therapy with Ara-C and VLB was especially effective against proliferating B lymphs. The results of this study seem to suggest that the drugs also significantly lower the percent of T lymphs in the spleen and lymph nodes. The effect of the leucogenenol seems to be to counteract some of the cytotoxic affects of the drugs. In the spleen this was shown by the slightly significant increase in percent B lymphs and no change in the T lymphs. Also the early increases in the percent B lymphs of the lymph nodes would suggest leucogenenols counteracting effects on the drugs. It is probable that the significant decreases in spleen and liver weight to total body weight ratios would have meant prolonged survival times which would have correlated with the results of Averbeck (1973).

Future work in this area should include several changes. First of all, more mice should be sacrificed per

animal group per week. This would enable better statistical analysis. Secondly, an animal group given FV + Ara-C + VLB should be included. More statements could then be made as to the effects of the chemotherapy and the counteracting effects of the leucogenenol. Further studies with leucogenenol might be done to monitor its effects on graft versus host reactions and its effect on solid tumors such as sarcomas and carcinomas.

SUMMARY

The purpose of this study was to quantitate the effects of leucogenenol and chemotherapy on the B and T lymphocyte populations in FV infected mice. The effects of leucogenenol seem to be the stimulation of both B and T lymphocyte stem cell lines. This was most clearly shown by the increase in the number of identifiable B and T lymphocytes in the peripheral blood. The effects of the leucogenenol was also to increase lymphoid cell counts and to decrease percents of B cells in the spleen and lymph nodes. The drugs did offset the effects of the FV only but the addition of leucogenenol seems to work in opposition to the drugs by reducing their leucopenic effects.

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